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13. ABSTRACT (Maximum 200 words) This proposal is designed to investigate the role of PTHrP in breast cancer-mediated osteolysis. Observations in patients with bone metastases suggest that breast cancer cells in bone express PTHrP more frequently than in soft tissue sites of metastasis or in the primary tumor. Three isoforms of PTHrP, 1-139, 1-141 and 1-173, are products of alternative splicing in humans, but the specific contribution of each of these isoforms to osteolytic metastasis caused by breast cancer has not been evaluated. To determine the role of these isoforms in breast cancer metastasis to bone, the human breast cancer cell line MDA-MB-231 (MDA-231) was stably transfected with similar amounts of cDNAs for human prepro PTHrP-(1-139), -(1-141) or -(1-173), driven by a CMV promoter, and studied in a model of human breast cancer metastasis to bone. Conditioned media from stable MDA/PTHrP-(1-139) clones contained significantly more PTHrP, compared with MDA/PTHrP-(1-141), -(1-173) or parental MDA-231. Nude mice were inoculated into the left cardiac ventricle with MDA-231/ PTHrP-(1-139), -(1-141), -(1-173) clones or parental MDA-231 cells. Osteolytic lesion area of radiographs was greatest in mice bearing MDA/PTHrP-(1-139) compared with those bearing MDA/PTHrP-(1-141), -(1-173) or parental MDA-231. Ca ⁺⁺ was significantly higher in the MDA/PTHrP-(1-139) compared with the MDA/PTHrP-(1-141), -(1-173) or parental MDA-231 groups as was the plasma PTHrP concentration. The data demonstrate that overexpression of PTHrP-(1-139) isoform in the human breast cancer cell line MDA-MB-231 results in greater PTHrP secretion <i>in vitro</i> and enhanced osteolysis with increased plasma PTHrP concentrations and hypercalcemia <i>in vivo</i> compared with overexpression of PTHrP-(1-141) or -(1-173). Differential cell processing of the isoforms may result in more efficient secretion of PTHrP-(1-139) and the osteolysis that is characteristic of breast cancer				
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INTRODUCTION

NATURE OF THE PROBLEM AND BACKGROUND OF PREVIOUS WORK

Breast Cancer, Hypercalcemia and Osteolysis

Breast cancer is associated with significant morbidity in the skeleton. Specifically, breast cancer can involve bone through both metastatic and humoral mechanisms. Metastases to bone are more commonly osteolytic than osteoblastic and are responsible for the complications of bone pain, pathologic fracture, hypercalcemia and nerve compression syndromes that many breast cancer patients suffer from (1). Eighty-four per cent of patients dying of breast cancer have bone metastases (2).

Hypercalcemia is commonly associated with breast cancer, occurring in up to 40% of afflicted women during the course of their disease (2,3). Skeletal destruction by metastatic tumor has been felt to be the major mechanism responsible for hypercalcemia (3). Increased osteoclastic bone resorption in areas surrounding breast cancer metastasis has been documented histologically (4,5) suggesting that factors secreted by breast cancer cells can locally activate osteoclasts. Recent evidence, however, suggests that osteolytic bone metastasis may not be the only mechanism responsible for breast cancer hypercalcemia and that humoral mechanisms may contribute in as much as 30-60% of the cases (6-8). In one study, 15% of 147 hypercalcemic breast cancer patients had no bone metastases (9).

PTHrP and Breast Cancer

Parathyroid hormone-related protein (PTHrP) is a major mediator of humoral hypercalcemia of malignancy, due to its PTH-like actions. This protein was purified in 1987 from human lung cancer (10), breast cancer (11) and renal cell carcinoma (12) simultaneously by several independent groups. Cloning and expression followed shortly thereafter (13).

PTHrP has since been extensively studied and found to have many similarities to PTH. It has 70% homology to the first 13 amino acids of the N-terminal portion of PTH (13), binds to PTH receptors (14) and shares similar biologic activity to PTH (15). Specifically, it stimulates adenylate cyclase in renal and bone systems (11,12,15-17), increases renal tubular reabsorption of calcium and osteoclastic bone resorption (16,17), decreases renal phosphate uptake (15,16,18) and stimulates 1α -hydroxylase (15). PTHrP has been found in a variety of tumor types as well as normal tissue (19-22). The widespread expression of PTHrP in normal as well as malignant tissue was the first evidence that the hormone has a role in normal physiology. In addition to the PTH-like effects, PTHrP has many non-PTH-like properties (23), some of which include regulation of placental calcium transport (22), possible establishment of bone metastasis in breast cancer (24,25), and autocrine regulation of the growth of some tumors (26). The regulation of PTHrP is poorly understood, but factors such as prolactin (27), glucocorticoids, $1,25(\text{OH})_2\text{D}_3$ (28), epidermal growth factor (28), $\text{TGF}\alpha$ (29), $\text{TGF}\beta$ (30), estrogen (31-34) and stretch (35) have been shown to regulate gene expression and extracellular calcium concentration has been shown to control the production of PTHrP in vitro in Leydig tumor cells (36).

It is now clear that PTHrP is a significant factor in mediating hypercalcemia in breast

cancer (37). One of the 3 tumors from which PTHrP was originally purified was a breast cancer from a patient with humoral hypercalcemia of malignancy (11). PTHrP was detected by immunohistochemical staining in 60% of 102 invasive breast tumors removed from normocalcemic women, but not in normal breast tissue (24). By immunohistochemistry (25) and in situ hybridization (38), it was detected in 12 of 13 breast cancer metastases in bone prompting speculation that production of PTHrP as a bone-resorbing agent may contribute to the ability of breast cancers to grow as bone metastasis. Along these lines, Bundred and colleagues found positive immunohistochemical staining for PTHrP in 56% of 155 primary breast tumors from normocalcemic women and PTHrP positivity was related to the development of bone metastases (39). Additionally, 65-92% of hypercalcemic breast cancer patients (with and without bone metastasis) had detectable plasma PTHrP concentrations by radioimmunoassay (RIA) similar to those documented in patients with humoral hypercalcemia of malignancy due to non-breast tumors (40,41).

PTHrP in Nonmalignant Breast Disease

In addition to its role in malignancy, PTHrP is important in the normal physiology of breast (42). It is expressed in lactating mammary tissue (43) and secreted into milk at concentrations 10,000-100,000 times greater than plasma concentrations of humans with malignancy-associated hypercalcemia (44-48). Suckling increases PTHrP gene expression and this appears to be mediated through prolactin (49). Estrogen has been shown to increase PTHrP expression in uterine tissue and *in vitro* studies suggest that there may be estrogen response elements present in the PTHrP gene (50-53). Increased plasma PTHrP concentrations have been described in at least 2 patients with the rare syndrome of lactational hypercalcemia (54-56). Animal studies have demonstrated a PTHrP gradient across the mammary gland in lactating goats (48) indicating that PTHrP may gain access to the maternal circulation during lactation. In support of this, a recent clinical study has shown detectable plasma PTHrP concentrations in 63% of breast-feeding mothers while similar measurements in bottle-feeding control mothers were undetectable (57). Thus, PTHrP may be responsible for mobilizing calcium from maternal bone for use in milk production and it may be the implicating factor in lactation-associated bone loss (58).

PTHrP as a Growth Regulator

PTHrP is produced in relatively low concentrations in breast myoepithelial cells (59). A transgenic mouse model, in which PTHrP is over expressed in skin and breast myoepithelial cells through the use of a human keratin promoter, has demonstrated breast hypoplasia. Specifically, female transgenics had a severe reduction in the number of albeit normal terminal ducts and acini in the breast suggesting that PTHrP may play a role in regulating ductular proliferation and/or differentiation during mammaryogenesis (60). These mice also had failure of normal hair follicle development indicating a similar role for PTHrP in the skin.

Along those lines, disruption of PTHrP expression in a normal keratinocyte cell line, using antisense technology, results in enhanced growth of the cells in culture (61). *In vivo*, homozygous mice for the PTHrP null mutation are born with a multitude of skeletal abnormalities, including defects in the bone growth plate (62). These findings, along with those of the above described transgenic mice, suggest that either over- or under- expression of PTHrP in normal cells result in abnormalities of growth and possibly differentiation.

In malignant cells, PTHrP has been shown to act as an autocrine growth factor in a renal cell carcinoma cell line (26) and more recently, in a squamous cell carcinoma line (63). There are no reported studies on the role of PTHrP as an autocrine growth factor in breast cancer.

Regulation of PTHrP by Other Tumor-associated and Bone-derived Growth Factors

Other tumor-associated growth factors as well as bone-derived growth factors may be important regulators of PTHrP expression in both malignant and non-malignant tissue. Epidermal growth factor has been shown to increase PTHrP expression in a keratinocyte cell (64) line while TGF- α , a breast cancer tumor product (65), enhances PTHrP expression in a human squamous cell carcinoma of the lung (29). Moreover, other tumor-associated factors may modulate the end organ effects of PTHrP. TGF- α enhances the hypercalcemic effects of PTHrP in an animal model of malignancy-associated hypercalcemia (66) and it can modulate the renal and bone effects of PTHrP as well (67,68). Additionally, TGF- β , which is present in high concentrations in the bone microenvironment, has been shown to enhance secretion of and stabilize the message for PTHrP in a renal cell carcinoma (30) as well as in an epidermal squamous cell carcinoma (69).

Implications of PTHrP Status in Breast Cancer

These findings have important implications for the ability of breast cancer to affect the skeleton. First, breast cancers expressing PTHrP in addition to other tumor-associated factors, such as TGF- α (65), may be more likely to affect the skeleton through humoral and osteolytic mechanisms if the co-expressed factor enhances PTHrP expression in the primary tumor. Second, if estrogen regulates PTHrP expression in breast cancer cells as it does in other tissues, estrogen receptor positive tumors may preferentially express PTHrP. Finally, growth of breast cancer cells in bone may be enhanced if the tumor cells express PTHrP. TGF- β , as well as other bone derived growth factors, are present in high concentration in the bone microenvironment (70) and are released from bone during the process of osteoclastic bone resorption (71). PTHrP expression in breast cancer cells lodged in bone is likely to be increased in the presence of TGF- β . In this scenario, osteoclastic bone resorption is increased further causing release of more TGF- β and other growth factors into the bone microenvironment leading to further enhancement of PTHrP expression in the breast cancer cells. If PTHrP acts as an autocrine growth factor in breast cancer cells, as it does in some tumor models, then tumor growth would be enhanced as well. The clinical findings of an increased incidence of PTHrP expression in bone compared with other sites by Powell and colleagues (25,38) supports the notion that production of PTHrP as a bone resorbing agent may contribute to the ability of breast cancers to grow as bone metastases.

If PTHrP expression in the primary breast tumor indicates a propensity to metastasize to bone due to its potent bone resorbing capability, early treatment with inhibitors of bone resorption is likely to prevent or delay the development of bone metastases as well as reduce the catastrophic complications of pain, hypercalcemia, fracture and nerve compression syndromes. It is already clear from clinical studies that the use of bisphosphonates, potent inhibitors of bone resorption, significantly reduces skeletal morbidity in advanced breast cancer (72-74). Bisphosphonates have also been shown to decrease the number of bone metastases in animal models (75,76), but it is unclear whether or not these tumors express PTHrP. However, since the safety of long term bisphosphonate use has not been determined and bone mineralization defects can occur with high doses of these drugs, it would be of benefit, as well as cost effective, to identify which patients are at risk to develop bone metastases and treat only those rather than treat all women with breast cancer. The clinical evidence thus far supports PTHrP as a marker to identify such women, but better animal models are needed to clarify this role.

Knowledge of PTHrP status may also have significant therapeutic implications in treating breast cancer-associated hypercalcemia. Although hypercalcemia in breast cancer is often associated with bone metastases, it is clear that humoral mechanisms may contribute in as much as 60% of the cases. Traditionally, treatment has been directed toward inhibiting bone resorption and this is often effective. However, it has now become evident that bisphosphonate therapy is less effective in patients with higher plasma concentrations of PTHrP and without radiological evidence of bone metastases (77,78). Thus, inhibition of bone resorption is effective when the major mechanism for hypercalcemia is increased bone resorption. Since PTHrP causes hypercalcemia by both increasing osteoclastic bone resorption and increasing renal tubular reabsorption of calcium, drugs that inhibit bone resorption alone may not normalize the calcium concentration if the plasma PTHrP concentration is high enough to add a significant renal component to the hypercalcemia. Drugs directed against either the actions of PTHrP or the secretion of PTHrP may therefore be more beneficial in the bisphosphonate resistant situation. Unfortunately, no such drugs are available at the current time but the need for them is obvious. A potentially useful therapy may prove to be the use of monoclonal antibodies against PTHrP. Sato has recently described successful use of an anti-PTHrP-(1-34) monoclonal murine antibody in an animal model of humoral hypercalcemia that ameliorated hypercalcemia and prolonged survival time in severely ill animals (79).

In Vivo Models of Hypercalcemia and Osteolysis

These observations demonstrate the need for further study of the role of PTHrP in malignant and nonmalignant breast disease. The only research done to date on PTHrP expression in human breast cancer and its potential role in humoral hypercalcemia and the development of osteolytic bone metastases have involved the small clinical studies described above (24,25,39-41). Despite numerous animal models of human breast cancer (80) that have been described to date, human breast cancer cell lines have not been studied *in vivo* for PTHrP expression and its relationship to the development of osteolytic bone metastases and humoral hypercalcemia. Most animal models of breast cancer have been used to evaluate the effect of various factors (81-83) on breast cancer growth. Only one spontaneous rat mammary tumor (Walker 256 carcinosarcoma) has been shown to cause humoral hypercalcemia in rats (84), produce PTHrP (85) and cause osteolytic bone metastases (75). Given the accumulating evidence documenting a humoral mechanism for hypercalcemia in breast cancer, the established role of PTHrP in humoral hypercalcemia of malignancy, the presence of PTHrP in malignant as well as lactating breast tissue and the presence of PTHrP in established breast cancer metastases to bone, it is evident that established models of human breast cancer should be evaluated for PTHrP expression and its relationship to the skeleton. Using animal models will be beneficial in defining this aspect of the pathophysiology of breast cancer and this will in turn have important prognostic and therapeutic implications.

Historically, it has been difficult to produce bone metastases in animal models of malignancy. Tumors inoculated subcutaneously or intramuscularly do not metastasize in nude mice and tumors inoculated into the tail vein usually produce only lung metastases. Yoneda has developed an animal model of human breast cancer cell metastasis to bone (76,86) which is based on a model originally described by Arguello (87). In this model, MDA-MB-231 breast cancer cells injected into the left ventricle of nude mice reliably produce osteolytic lesions that are evident radiologically as well as histologically. This model has been used to show that the bisphosphonate, risedronate, decreased osteolytic lesions when given simultaneously with tumor cells and inhibited both an increase in new bone metastases and progression of each metastatic focus when given to animals with pre-existing osteolytic lesions (76).

PURPOSE OF PRESENT WORK

Breast cancer affects the skeleton through humoral and local osteolytic mechanisms to cause the devastating complications of hypercalcemia, pain, fracture and nerve compression syndromes. PTHrP is an important humoral mediator of hypercalcemia in cancer and may have physiologic roles in the lactating breast as well as in cell growth and differentiation. The role of PTHrP in the pathophysiology of breast cancer is significant for several reasons. 1) PTHrP mediates hypercalcemia through its systemic effects of increasing osteoclastic bone resorption as well as renal tubular calcium reabsorption in at least 50% of hypercalcemic breast cancer patients even in the presence of bone metastases. 2) Due to its potent bone resorbing capacity, PTHrP expression in the primary tumor may aid in establishment of the bone metastases that are so characteristic of patients with breast cancer. 3) Growth factors present in the bone microenvironment further enhance PTHrP expression in breast cancer cells present in bone and promote development of osteolytic lesions and tumor growth. Thus, PTHrP expression in the primary breast tumor may be a marker for the development of hypercalcemia and bone metastases.

The purpose of this study is to define the role of PTHrP in the pathophysiology of breast cancer using animal models of breast cancer-mediated humoral hypercalcemia and osteolytic bone metastases. Our previous studies reported in the first 2 years of this proposal indicate that 50% of breast cancer cell lines tested secrete low, but significant amounts of PTHrP. Over-expression of PTHrP-(1-141) increased osteolytic metastasis in a mouse model of human breast cancer metastasis to bone. Furthermore, treating mice with a neutralizing antibody to PTHrP inhibited the development of new bone metastasis and the progression of established bone metastasis caused by MDA-MB-231, a human breast cancer cell line which makes low amounts of PTHrP. Since it is now clear that PTHrP has an important role in the development and progression of breast cancer metastasis to bone, our aim was to investigate the role of the various isoforms of PTHrP in the pathophysiology of breast cancer metastasis to bone.

The human PTHrP gene is complex and spans approximately 15 kilobases of genomic DNA and is composed of nine exons (99). Three isoforms of PTHrP, 1-139, 1-141 and 1-173, are products of alternative splicing in humans and depend on whether exon VI is spliced to exon VII, VIII, or IX respectively. The gene is under the control of three distinct promoters and agents which regulate PTHrP expression such as transforming growth factor β (TGF β), glucocorticoids and epidermal growth factor act at least in part by altering the rate of gene transcription (99). However, the specific contribution of each PTHrP isoform to osteolytic metastasis caused by breast cancer or hypercalcemia is not known.

To determine the role of these isoforms in breast cancer metastasis to bone, the human breast cancer cell line MDA-MB-231 (MDA-231) was stably transfected with similar amounts of cDNAs for human prepro PTHrP-(1-139), -(1-141) or -(1-173), driven by a CMV promoter, and studied in a model of human breast cancer metastasis to bone. The results presented here demonstrate that although breast cancer cells expressing the PTHrP-(1-139) isoform had similar in vitro growth rates as those expressing the other isoforms or the parental MDA-MB-231 cells, PTHrP secretion was markedly increased. This was associated with enhanced osteolysis and hypercalcemia when the cells were studied in a mouse model of human breast cancer metastasis to bone. Information gained from these studies will have important prognostic and therapeutic implications.

METHODS OF APPROACH

In order to define the role of PTHrP in the pathophysiology of breast cancer-associated hypercalcemia and skeletal complications in a systematic fashion, the following objectives were originally proposed.

1. **SPECIFIC AIM #1: To screen known breast cancer cell lines for PTHrP expression and secretion and to determine if PTHrP expression is related to estrogen receptor status.**
 - a. Known breast cancer cell lines (both estrogen receptor positive and negative) will be grown in culture along with positive and negative controls. Media conditioned for 24 hours will be screened for PTHrP immunoreactivity by immunoradiometric assay.
 - b. RNA will be isolated from above cell lines in the presence and absence of estrogen and PTHrP expression will be determined using Northern analysis.
2. **SPECIFIC AIM #2: Determine if known human breast cancer cell lines will cause humoral hypercalcemia and if this is PTHrP-mediated.**
 - a. Measure standard parameters of calcium homeostasis in nude mice bearing human breast tumors.
 - b. Determine that hypercalcemia observed in mice bearing PTHrP+ breast tumors is PTHrP-mediated. Two approaches will be used: i) to decrease PTHrP secretion by transfecting PTHrP + lines with PTHrP antisense ii) decrease PTHrP effects by administration of neutralizing antibody.
 1. Transfection of PTHrP antisense cDNA into breast cancer cell lines that secrete PTHrP and cause hypercalcemia in nude mice.
 2. Measurement of Ca^{++} in mice bearing hypercalcemic PTHrP+ breast cancer cell lines that have been transfected with PTHrP antisense cDNA.
 3. Measurement of Ca^{++} in mice bearing hypercalcemic PTHrP+ breast cancer cell lines that are treated with anti-PTHrP-(1-34) monoclonal antibody.

3. **SPECIFIC AIM #3: To determine the role of PTHrP in the development of osteolytic metastases in breast cancer.**
- a. Is PTHrP expression enhanced in the bone microenvironment relative to other metastatic sites? Using an animal model of breast cancer-mediated osteolysis, PTHrP expression will be compared in bone and non-bone sites using immunohistochemistry and in situ hybridization.
 - b. Does expression of PTHrP in the primary tumor enhance the development and quantity of osteolytic bone metastases? Breast cancer cell line, MDA-231 will be transfected with the cDNA for human PTHrP or PTHrP-AS (antisense orientation as a control) and used in the osteolytic model.
 - 1. Production of stable MDA-231 clones expressing PTHrP or PTHrP-AS by calcium phosphate precipitation.
 - 2. Effect of MDA-231/PTHrP on development of osteolytic bone metastases will be assessed by inoculating these cells into the left ventricle of mice and determining if the quantity and size of the bone metastases differ from similarly inoculated control MDA-231/PTHrP-AS. Neutralizing antibodies will be given to attempt to block osteolysis in mice inoculated with MDA-231/PTHrP cells.

BODY

METHODS

Cell culture

MDA-MB-231 cells (13) were (provided by C. Kent Osborne, M.D.) were cultured in DMEM (Life Technologies, Grand Island, NY) containing 10% FCS (Hyclone, Logan, UT), 1% penicillin/streptomycin and nonessential amino acids (Gibco, Gaithersburg, MD) in a 37°C atmosphere of 5% CO₂/air. To test the effect of TGFβ on PTHrP secretion by MDA-MB-231 cells, 10⁴ cells/mL were plated onto 48 well plates. When near confluence, cells were washed with phosphate buffered saline (PBS) and 250 µl of serum-free DMEM containing TGFβ1 (5 ng/mL) was added to each well. TGFβ1 was purchased from R & D, Minneapolis, MN. Conditioned media were collected after 48 hours and stored at -70°C for PTHrP measurement. Cell number was counted for each well to correct the PTHrP concentration of the conditioned media. Triplicate measurements were performed.

To determine the growth rate of MDA-MB-231 cells and respective clones expressing the PTHrP-(1-139), -(1-141) or -(1-173) isoforms, 10⁴ cells/mL were plated onto each of 24-well plates. Cell number was counted every day for eight days and each measurement was performed in triplicate.

Stable transfection of MDA-MB-231 cells with cDNA for human prepro PTHrP-(1-139), -(1-141) or -(1-173)

The pcDNA3/PTHrP-(1-139), -(1-141), -(1-173) or the empty vector pcDNA3 was transfected into MDA-MB-231 cells by calcium phosphate precipitation (Sambrook). Single clones were isolated by limiting dilution in the presence of the selective marker, G418 (Sigma, St. Louis, MO). Clones were screened by measuring the amount of secreted PTHrP in serum-free 48-hour conditioned media.

***In vivo* experiments**

Animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Female nude mice 4-6 weeks of age were housed in laminar flow isolated hoods with 12 hour light/12 hour dark cycle. Water supplemented with vitamin K and autoclaved mouse chow were provided ad libitum.

Whole blood samples for ionized calcium concentration were obtained by retro-orbital puncture under metofane anesthesia. Blood samples for PTHrP measurement were similarly obtained and collected on ice in vacutainer tubes containing EDTA (Becton Dickinson, NJ) and 400 IU/mL aprotinin (Sigma, St. Louis, MO).

Tumor inoculation into the left cardiac ventricle was performed while the mice were anesthetized with a ketamine/xylazine mixture and positioned ventral side up based on a modification of Arguello (87). The left cardiac ventricle was punctured percutaneously using a 27 gauge needle attached to a 1 mL syringe containing suspended tumor cells. Visualization of bright red blood entering the hub of the needle in a pulsatile fashion indicated correct position in the left cardiac ventricle.

Experimental protocols

Bone metastasis

Mice were inoculated with tumor cell suspensions of MDA/PTHrP-(1-139), MDA/PTHrP-(1-141), MDA/PTHrP-(1-173) or MDA/pcDNA3 cells into the left cardiac ventricle (n=7 per group) on day 0. Baseline radiographs and body weights as well as blood for Ca²⁺ and plasma PTHrP concentrations were obtained at this time. Radiographs were taken on day 21 and then weekly until sacrifice to monitor progression of osteolytic metastasis. Ca²⁺ and body weight were measured weekly for three weeks post tumor inoculation until sacrifice, at which time most mice

in the control groups were cachectic and paraplegic. At the time of sacrifice, blood was collected for Ca^{2+} and PTHrP measurement, and all bones and soft tissues were harvested and fixed in formalin for histologic analysis. Autopsy was performed on all mice, and those with tumor in the chest were excluded from analysis, as this indicated that part or all of the tumor inoculum did not properly enter the left cardiac ventricle.

Analytical Methods

Ca^{2+} measurement

Ca^{2+} concentrations were measured in whole blood using a Ciba Corning 634 ISE Ca^{2+} /pH analyzer (Medfield, MA) and adjusted using the internal algorithm of the instrument to pH 7.4. Samples were run in duplicate and the mean value recorded.

PTHrP Assay

PTHrP concentrations were measured in conditioned media and plasma using a 2-site immunoradiometric assay (Nichols Institute, San Juan Capistrano, CA) which uses 2 polyclonal antibodies that are specific for the N-terminal -(1-40) and -(60-72) portions of PTHrP and has a calculated sensitivity of 0.3 pmol/L (90). PTHrP concentrations in conditioned media samples were calculated from a standard curve generated by adding recombinant PTHrP-(1-86) to the specific type of medium (unconditioned) used and were considered undetectable if media concentrations were <0.3 pmol/L prior to correction for cell number.

Radiographs and measurement of osteolytic lesion area

Animals were x-rayed in a prone position against the film (22 x 27cm X-Omat AR, Kodak) and exposed with x-rays at 35 KVP for 6 seconds using a Cabinet X-ray system-Faxitron Series, Hewlett-Packard (Model 43855A), (Faxitron X-ray Corporation, Buffalo Grove, IL). All radiographs were evaluated in blinded fashion. The area of osteolytic bone metastases was calculated using a computerized image analysis system. Video images of radiographs were captured using a frame grabber board (Targa+, Truevision, Inc., USA) on a PC system. Quantitation of lesion area was performed using image analysis software (Java, Jandel Video analysis, Jandel Scientific, CA).

Statistical analysis

Results are expressed as the mean \pm the standard error of the mean. Data were analyzed by repeated measures analysis of variance followed by Tukey-Kramer post test. P values of <0.05 were considered significant.

RESULTS

Stable clones expressing the cDNA for the human preproPTHrP-(1-139), -(1-141), or (1-173) secreted different amounts of PTHrP as detected by IRMA of serum-free conditioned media (Figure 1). All transfectants secreted significantly more PTHrP than the parental MDA-MB-231 cells. Those MDA-MB-231 cells expressing PTHrP-(1-139) consistently secreted the most PTHrP compared with the other isoforms. MDA-MB-231 cells expressing the PTHrP-(1-173) isoform secreted more PTHrP than those expressing the PTHrP -(1-141) isoform. This pattern of secretion was similar in transient transfections of 293 cells and suggests differential processing of the 1-139 isoform. All transfectants, except those expression the 1-139 isoform responded to TGF β with an increase in PTHrP secretion. *In vitro* growth rates were similar for all transfectants and did not differ from the parental MDA-MB-231 cells (Figure 2).

In vivo, bone metastases developed in all groups tested: parental MDA-MB-231, PTHrP-(1-139), -(1-141) and -(1-173). Mice bearing the MDA/PTHrP-(1-139) developed strikingly larger bone metastases which occurred much earlier than those of mice bearing the MDA/PTHrP-(1-173) or -(1-141) (Figure 3). These differences were statistically significant as quantitated by computerized image analysis of radiographs (Figure 4). The total lesion area on radiographs was significantly larger in the mice bearing MDA/PTHrP-(1-139) compared with mice bearing parental MDA-MB-231, MDA/PTHrP-(1-173) and MDA/PTHrP-(1-141). The latter 3 groups did not differ significantly with regard to lesion area. Mice bearing MDA/PTHrP-(1-139) had more lesions at day 48 compared with mice in the other groups (Figure 4). The lesion number in mice bearing parental MDA-MB-231, MDA/PTHrP-(1-173) and MDA/PTHrP-(1-141) reached values comparable to those of the MDA/PTHrP-(1-139) group, however, this was significantly longer after tumor inoculation.

Significant hypercalcemia was evident in mice bearing MDA/PTHrP-(1-139) tumors compared with those bearing the -(1-173), -(1-141) or parental MDA-MB-231 cells (Figure 5). This was due to a marked increase in the plasma PTHrP concentration in the MDA/PTHrP-(1-139) group determined at sacrifice. The PTHrP concentrations were similar to those observed in humans with malignancy-associated hypercalcemia (Burtis). Mice in all other groups remained normocalcemic throughout the experimental period. Plasma PTHrP concentrations were significantly higher in the mice bearing MDA/PTHrP-(1-173) and -(1-141) at the time of sacrifice when compared with those taken at baseline. However, these concentrations were not increased to the degree with which systemic effects of PTHrP should be observed. Additionally mice bearing the MDA/PTHrP-(1-139) tumors lost significantly more body weight than mice in the other groups.

In these experiments, metastasis to sites other than bone included adrenal gland, ovary, lung and liver in all groups. However, there were no significant differences in metastases to such nonbone sites between any of the groups.

DISCUSSION

The data presented here demonstrate that overexpression of PTHrP-(1-139) in the human breast cancer cell line, MDA-MB-231, is associated with enhanced PTHrP secretion *in vitro* compared with other isoforms of PTHrP-(1-173) and -(1-141). This enhanced PTHrP secretion was also evident *in vivo* as mice bearing the MDA/PTHrP-(1-139) tumors had increased plasma PTHrP concentrations that were significantly different from those at baseline or those at sacrifice in mice bearing parental MDA-MB-231, MDA/PTHrP-(1-173) or MDA/PTHrP-(1-141). The enhanced PTHrP secretion by MDA-MB-231 cells *in vitro* and *in vivo* correlated to increased bone destruction and hypercalcemia in a mouse model of human breast cancer metastasis to bone.

These findings are consistent with the previous clinical and experimental evidence which implicate PTHrP as a mediator of the local bone destruction associated with breast cancer metastasis to bone. PTHrP is present in tumor cells in bone in the majority of patients with advanced breast cancer (25,38), and development of subsequent bone metastases is positively correlated with PTHrP expression in the primary site (39,40). Moreover, neutralizing antibodies to PTHrP abrogate the osteolytic bone lesions in a mouse model of human breast cancer metastases to bone (98). Finally, treatment with bisphosphonates, potent inhibitors of osteoclastic bone resorption are associated with decreased morbidity in patients with breast cancer metastases to bone (100).

Current evidence suggests that PTHrP expression by human breast cancer cells is enhanced in the bone microenvironment (25). Growth factors released in active form when bone is resorbed such as transforming growth factor β (TGF β) (101) may enhance PTHrP production in that site. In these experiments parental MDA-MB-231 cells or those expressing the -(1-141) or -(1-173) isoforms significantly increased PTHrP production in response to TGF β . Since the expression of the cDNAs are driven by a constitutive promoter, it is likely that the effects of TGF β are post-transcriptional. One known effect of TGF β is to stabilize the messenger RNA for PTHrP and this has been demonstrated in a renal cell carcinoma (102). The MDA-MB-231 cells expressing the PTHrP-(1-139) isoform did not respond to TGF β by increasing PTHrP production. The reasons for this consistent finding are not clear but may be due to the possibility that the MDA/PTHrP-(1-139) cells are secreting the maximal amount of PTHrP and cannot be further stimulated.

The exact role of the different isoforms of PTHrP in cancer and in normal physiology are not known. Additionally, whether any isoform predominates in malignancy is unknown. Previous work has demonstrated that all isoforms are present in the malignant cell lines BEN, COLO 16, HcCaT, MCF7, MDA-MB-231 and T-47D at the mRNA level and that expression is cell-specific with regulator-induced promoter usage (99). What is clear from the work presented here is that overexpression of the PTHrP-(1-139) isoform results in more efficient secretion of PTHrP from MDA-MB-231 cells as well as from a small number of other cell lines which have been tested such as HEK 293 cells and the human breast cancer cell line, MCF7. This efficient secretion of PTHrP results in the enhanced osteolysis and hypercalcemia observed in this mouse model of bone metastases. Whether this enhanced capacity to cause bone metastasis is an isoform-specific property rather than due to an absolute increase in PTHrP production alone cannot be determined from these studies.

Another consideration is that the different PTHrP isoform proteins are processed into different peptides which have different actions. Like PTH and other endocrine peptides, PTHrP undergoes endoproteolytic posttranslational processing that results in several secretory forms: 1) an amino-terminal PTHrP-(1-36), 2) a mid-region species that begins at amino acid 38 that

has an undefined carboxyl terminus (104,105) and 3) a carboxyl-terminal species that is recognized by an antibody directed against the 109-138 region (104-106). The preponderance of and arrangement of basic residues in the protein sequence suggest that members of the subtilisin family of endoproteases such as furin (105), PC 1/3, PC-2, PACE-4 and PC8 (106) are responsible for such processing (107,108). Posttranslational modification of PTHrP also occurs as glycosylation of an amino-terminal PTHrP species produced by keratinocytes has been reported (109). Regulation of PTHrP secretion may be cell-specific as PTHrP expressed in neuroendocrine cells is secreted in a regulated fashion as compared with a constitutive secretion when expressed in non-neuroendocrine cell types such as squamous cell carcinoma (108). Although PTHrP mediates its calcemic effects through the classical PTH/PTHrP receptor, there is evidence for a separate PTHrP receptor (110). However, the function of such a receptor remains unclear.

In this study, PTHrP concentrations were measured *in vitro* and *in vivo* using a 2-site immunoradiometric assay which detects PTHrP-(1-72). Thus, it is not clear exactly which processed forms of PTHrP are secreted by the respective transfectants. However, these results suggest that differential cell processing of the isoforms may result in more efficient secretion of PTHrP-(1-139) and the consequent osteolysis that is characteristic of breast cancer.

REFERENCES

1. Guise TA and Mundy GR. Breast cancer and bone. Current Opinion in Endocrinology and Metabolism. 2:548-555, 1995.
2. Aaron AD. The management of cancer metastatic to bone. JAMA 272(15):1206-1209, 1994.
3. Yoneda T, Sasaki A, Mundy GR. Osteolytic metastases in breast cancer. Breast Cancer Res Treat 32(1):73-84, 1994.
4. Galasko CSB. Mechanisms of bone destruction in the development of skeletal metastasis. Nature 263:507-508 1976
5. Taube T, Elomaa I, Blomqvist C, Beneton MNC, Kanis JA. Histomorphometric evidence for osteoclast-mediated bone resorption in metastatic breast cancer. Bone 15(2):161-166, 1994.
6. Isles C, Carcangiu ML, Stewart AF. Hypercalcemia in breast cancer: reassessment of the mechanism. Am J Med 82:1143-1146 1987
7. Kimura S, Adachi I, Yamaguchi K, Suzuki M, Shimada A, Sato Y, Nagaoka K, Abe K. Stimulation of calcium reabsorption observed in advanced breast cancer patients with hypercalcemia and multiple bone metastasis. Jpn J Cancer Res 76:308-314 1985
8. Gallacher SJ, Fraser WD, Patel U, Logue FC, Soukop M, Boyle IT, Ralston SH. Breast cancer-associated hypercalcemia: a reassessment of renal calcium and phosphate handling. Annals of Clinical Biochemistry 27:551-6 1990
9. Coleman RE, Fogelman I, Rubens RD. Hypercalcemia and breast cancer - an increased humoral component in patients with liver metastasis. Eur J Surg Oncol 14:423-428 1988
10. Moseley JM, Kubota M, Diefenbach-Jagger H, Wettenhall REH, Kemp BE, Suva LJ, Rodda CP, Ebeling PR, Hudson PJ, Zajac JD, Martin TJ. Parathyroid hormone-related protein purified from a human lung cancer cell line. Proc Natl Acad Sci USA 84:5048-5052, 1987
11. Burtis WJ, Wu T, Bunch C, Wysolmerski J, Insogna K, Weir E, Broadus AE, Stewart AF. Identification of a novel 17,000-dalton parathyroid hormone-like adenylate cyclase-stimulating protein from a tumor associated with humoral hypercalcemia of malignancy. J Biol Chem 262(15):7151-7156 1987
12. Strewler GJ, Stern P, Jacobs J, Eveloff J, Klein RF, Leung SC, Rosenblatt M, Nissenson R. Parathyroid hormone-like protein from human renal carcinoma cells structural and functional homology with parathyroid hormone. J Clin Invest 80:1803-1807, 1987
13. Suva LJ, Winslow GA, Wettenhall REH, Hammonds RG, Moseley JM, Dieffenbach-Jagger, Rodda C, Kemp BE, Rodriguez H, Chen E, Hudson PJ, Martin TJ, Wood WI. A parathyroid hormone-related protein implicated in malignant hypercalcemia: Cloning and expression. Science 237:893-896, 1987

14. Abou-Samra A, Juppner H, Force T, Freeman MW, Kong X, Schipani E, Urena P, Richards J, Bonventre JV, Potts JT, Kronenberg HM, Segre GV. Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat osteoblast-like cells: A single receptor stimulates intracellular accumulation of both cAMP and inositol triphosphates and increases intracellular free calcium. Proc Natl Acad Sci USA 89:2732-2736, 1992
15. Horiuchi N, Caulfield M, Fisher JE, Goldman M, Mckee R, Reagan J, Levy J, Nutt R, Rodan S, Schoefield T, Clemens T, Rosenblatt M. Similarity of synthetic peptide from human tumor to parathyroid hormone in vivo and in vitro. Science 238:1566-1568, 1987
16. Kemp BE, Moseley JM, Rodda CP, Ebeling PR, Wettenhall REH, Stapleton D, Dieffenbach-Jagger H, Ure F, Michelangeli BP, Simmons HA, Raisz LG, Martin TJ. Parathyroid hormone-related protein of malignancy: Active synthetic fragments. Science 238:1568-1570, 1987
17. Yates AJP, Gutierrez GE, Smolens P, Travis PS, Katz MS, Aufemorte TB, Boyce BF, Hymer TK, Poser JW, Mundy GR. Effects of a synthetic peptide of a parathyroid hormone-related protein on calcium homeostasis, renal tubular calcium reabsorption, and bone metabolism in vivo and in vitro in rodents. J Clin Invest 81:932-938, 1988
18. Sartori L, Weir EC, Stewart AF, Broadus AE, Mangin M, Barrett PQ, Insogna KL. Synthetic and partially-purified adenylate cyclase-stimulating proteins from tumors associated with humoral hypercalcemia of malignancy inhibit phosphate transport in a PTH-responsive renal cell line. J Clin Endocrinol Metab 66:459-461, 1987
19. Asa SL, Henderson J, Goltzman D, Drucker DJ. Parathyroid hormone-like peptide in normal and neoplastic human endocrine tissues. J Clin Endocrinol Metab 71:1112-1118, 1990
20. Thiede MA and Rodan GA. Expression of a calcium mobilizing parathyroid-like peptide in lactating mammary tissue. Science 242:161, 1988
21. Danks JA, Ebeling PR, Hayman J, Chou ST, Moseley JM, Dunlop J, Kemp BE, Martin TJ. Parathyroid hormone-related protein: Immunohistochemical localization in cancers and in normal skin. J Bone Min Res 4:273-278, 1989
22. Rodda CP, Kubota M, Heath JA, Ebeling PR, Moseley JM, Care AD, Caple IW, Martin TJ. Evidence for a novel parathyroid hormone-related protein in fetal lamb parathyroid glands and sheep placenta: comparisons with a similar protein in humoral hypercalcemia of malignancy. J Endocrinol 117:261-71, 1988
23. Mallette LE. The parathyroid polyhormones: New concepts in the spectrum of peptide hormone action. Endo Rev 12:110-117, 1991
24. Southby J, Kissin MW, Danks JA, Hayman JA, Mosely JM, Henderson MA, Bennett RC, Martin TJ. Immunohistochemical localization of parathyroid hormone-related protein in breast cancer. Can Res 50:7710-7716 1990
25. Powell GJ, Southby J, Danks JA, Stillwell RG, Hayman JA, Henderson MA, Bennett RC, Martin TJ. Localization of parathyroid hormone-related protein in breast cancer metastasis: increased incidence in bone compared with other sites. Can Res 51:3059-3061 1991

26. Burton PBJ, Moniz C, Klight D. Parathyroid hormone-related peptide can function as an autocrine growth factor in human renal cell carcinoma. Biochem Biophys Res Commun 167:1134-1138, 1990
27. Theide MA. The mRNA encoding a parathyroid hormone-like peptide is produced in mammary tissue in response to elevations in serum prolactin. Mol Endocrin 3:1443-1447, 1989
28. Kremer R, Karaplis AC, Henderson J, Gulliver W, Banville D, Hendy GN, Goltzman D. Regulation of parathyroid hormone-like peptide in cultured normal human keratinocytes. J Clin Invest 87:884-893 1991
29. Burton PBJ, Knight DE. Parathyroid hormone-related peptide can regulate growth of human lung cancer cells. FEBS 305:228-232, 1992
30. Zakalik D, Diep D, Hooks MA, Nissenson RA, Strewler GJ. Transforming growth factor β increases stability of parathyroid hormone-related protein messenger RNA. J Bone Min Res 7 (Suppl 1):104A, S118, 1992
31. Fukayama S, Tashjian AH. Direct modulation by estradiol of the response of human bone cells (SaOS-2) to human parathyroid hormone (PTH) and PTH-related protein. Endocrinology 124:397-401, 1989
32. Thiede MA, Harm SC, Hasson DM, Gardner RM. in vivo regulation of parathyroid hormone-related peptide messenger ribonucleic acid in the rat uterus by 17β -estradiol. Endocrinology 128:2317-2323, 1991
33. Weir E, Daifotos A, Dreyer B, Burtis W, Broadus A. Estrogen-responsive expression of the parathyroid hormone-related peptide gene by cultured primary myometrial cells. J Bone Min Res 6 (Suppl 1):598A, 1991
34. Thiede MA, Harm SC, Gardner RM. In vivo regulation of the parathyroid hormone-related protein by estrogens and antiestrogens. J Bone Min Res 6 (Suppl 1):599A, 1991
35. Daifotis AG, Weir EC, Dreyer BE, Broadus AE. Stretch-induced parathyroid hormone-related protein gene expression in the rat uterus. J Biol Chem (33):23455-8 1992
36. Rizzoli R and Bonjour JP. High extracellular calcium increases the production of a parathyroid hormone-like activity by cultured Leydig tumor cells associated with humoral hypercalcemia. J Bone Min Res 4:839-844, 1989
37. Heath DA. Parathyroid hormone related protein. Clinical Endocrinology 38:135-6 1993
38. Vargas SJ, Gillespie MT, Powell GJ, Southby J, Danks JA, Moseley JM, Martin TJ. Localization of parathyroid hormone-related protein mRNA expression and metastatic lesions by in situ hybridization. J Bone Min Res 7(8):971-980 1992
39. Bundred NJ, Walker RA, Ratcliffe WA, Warwick J, Morrison JM, Ratcliffe JG. Parathyroid hormone related protein and skeletal morbidity in breast cancer. European Journal of Cancer 28(2-3):690-92 1992

40. Bundred NJ, Ratcliffe WA, Walker RA, Cjoley S, Morrison JM, JRatcliffe JG. Parathyroid hormone related protein and hypercalcaemia in breast cancer. British Medical Journal 303(6816):1506-9 1991
41. Grill V, Ho P, Body JJ, Johanson N, Lee SC, Kukreja SC, Mosely JM, Martin TJ. Parathyroid hormone-related protein; elevated levels in both humoral hypercalcemia and hypercalcemia complicating metastatic breast cancer. J Clin Endo Met 73:1309-1315 1991
42. Ratcliffe WA. Role of parathyroid hormone-related protein in lactation. Clinical Endocrinology 37:402-404 1992
43. Thiede MA, Rodan GA. Expression of a calcium-mobilizing parathyroid hormone-like peptide in lactating mammary tissue. Science 242:278-280 1988
44. Budayr AA, Halloran BP, King JC, Diep D, Nissenson RA, Strewler GJ. High levels of parathyroid hormone-like protein in milk. Proc Natl Acad Sci USA 86:7183-7185 1989
45. Yamamoto M, Fisher JE, Thiede MA, Caulfield MP, Rosenblatt M, Duong LT. Concentrations of parathyroid hormone-related protein in rat milk change with duration of lactation and interval from previous suckling, but not with milk calcium. Endocrinol 130:741-747 1992
46. Khosla S, Johansen KL, Ory SJ, Obrien PC, Kao PC. Parathyroid hormone-related peptide in lactation and in umbilical cord blood. Mayo Clin Proc 65:1408-1418 1990
47. Stewart AF, Wu TL, Insogna KL, Milstone LM, Burtis WJ. Immunoaffinity purification of parathyroid hormone-related protein from bovine milk and human keratinocyte-conditioned medium. J Bone Min Res 6:305-311 1991
48. Ratcliffe WA, Thompson GE, Care AD, Peaker M. Production of parathyroid hormone-related protein by the mammary gland of the goat. Journal of Endocrinology 133:87-93 1992
49. Thiede MA. The mRNA encoding a parathyroid hormone-like peptide is produced in mammary tissue in response to elevations in serum prolactin. Mol Endo 3:1443-47 1989
50. Fukayama S, Tashjian AH. Direct modulation by estradiol of the response of human bone cells (SaOS-2) to human parathyroid hormone(PTH) and PTH-related protein. Endocrinol 124:397-401 1989
51. Thiede MA, Harm SC, Hasson DM, Gardner RM. In vivo regulation of parathyroid hormone-related peptide messenger ribonucleic acid in the rat uterus by 17 β -estradiol. Endocrinol 128:2317-2323 1991
52. Weir E, Daifotos A, Dreyer B, Burtis W, Broadus A. Estrogen-responsive expression of the parathyroid hormone-related peptide gene by cultured primary myometrial cells. J Bone Min Res 6:suppl 1 A598 1991
53. Thiede MA, Harm SC, Gardner RM. In vivo regulation of the parathyroid hormone-related protein by estrogens and antiestrogens. J Bone Min Res 6:suppl 1 A599 1991

54. Lepre F, Grill V, Danks JA. Hypercalcemia in pregnancy and lactation due to parathyroid hormone-related protein production. Bone Min 10:s317 1990
55. Khosla S, van Heerden JA, Garib H, Jackson IT, Danks J, Hayman JA, Martin TJ. Parathyroid hormone-related protein and hypercalcemia secondary to massive mammary hyperplasia. N Engl J Med 322(16):1157 1990
56. Lepre F, Grill V, Ho PWM, Martin TJ. Hypercalcemia in pregnancy and lactation associated with parathyroid hormone-related protein. N Engl J Med 328(9):666-667 1993
57. Grill V, Hillary J, Ho PMW, Law FMK, MacIsaac RJ, MacIsaac IA, Moseley JM, Martin TJ. Parathyroid hormone-related protein: a possible endocrine function in lactation. Clinical Endocrinology 37:405-410 1992
58. Strewler GJ and Nissenson RA. The parathyroid hormone-related protein as a regulator of normal tissue functions. Current Opinion in Endocrinology and Diabetes 286-292 1993
59. Seitz PK, Cooper KM, Ives KL, Ishizuka J, Townsend CM, Rajaraman S, Cooper CW. Parathyroid hormone-related peptide production and action in a myoepithelial cell line derived from normal human breast. Endocrinology 133:1116-1124 1993
60. Wysolmerski J, Daifotis A, Broadus, Milstone L, Philbrick W. Overexpression of PTHrP in transgenic mice results in breast hypoplasia. J Bone Min Res 8(Supplement 1):129A 1993
61. Kaiser SM, Laneuville P, Bernier SM, Rhim JS, Kremer K, Goltzman D. Enhanced growth of a human keratinocyte cell line induced by antisense RNA for parathyroid hormone-related peptide. J Biol Chem 267:13623-13628, 1992
62. Karaplis AC, Luz A, Glowacki J, Bronson RT, Tybulewicz VL Kronenberg HM, Mulligan RC. Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. Genes Dev 8:277-89 1994.
63. Moy AA, Kats Y, Vasavada R, Dann P, Burtis WJ, Philbrick WM, Orloff JJ. Parathyroid hormone-related protein (PTHrP) is a potent autocrine growth promoter in a squamous carcinoma cell line. J Bone Min Res 8(Supplement 1):235A 1993
64. Allinson ET, Drucker DJ. Parathyroid hormone-like peptide shares features with members of early response gene family: Rapid induction by serum growth factors and cyclohexamide. Can Res 52(11):3103-9 1992
65. Nagata N, Yasutomo Y, Kugai N, Matsura Y, Akatsu T, Su G, Yama K, Kinoshita T, Takatami O, Mamiya Y, Yamaguchi K. Parathyroid hormone-related protein and transforming growth factor activities in an extract from a breast cancer associated with humoral hypercalcemia of malignancy. Jpn J Clin Oncol 19:353-359, 1989
66. Guise TA, Yoneda T, Yates AJP, Mundy GR. The combined effect of tumor produced parathyroid hormone-related protein and transforming growth factor α enhance hypercalcemia in vivo and bone resorption in vitro. J Clin Endocrinol Metab 77:40-45 1993
67. Pizurki L, Rizzoli R, Caverzasio J, Bonjour J. Effect of transforming growth factor α and

- parathyroid hormone-related protein on phosphate transport in renal cells. Am J Physiol 259:F929-F935, 1990
68. Pizurki L, Rizzoli R, Caverzasio J, Bonjour J. Stimulation by parathyroid hormone-related protein and transforming growth factor α of phosphate transport in osteoblast-like cells. J Bone Min Res 6:1235-1241, 1991
 69. Kiriyaama T, Gillespie MT, Glatz, JA, Fukumoto S, Moseley JM, Martin TJ. Transforming growth factor β stimulation of parathyroid hormone-related protein (PTHrP): a paracrine regulator? Molecular and Cellular Endocrinology 92:55-62 1992
 70. Hauschka PV, Mavrakos AE, Iafrati MD, Doleman SE, Klagsbrun M. Growth factors in bone matrix. J Biol Chem 261:12665-12674 1986
 71. Pfeilschifter J, Mundy GR. Modulation of transforming growth factor β activity in bone cultures by osteotropic hormones. Proc Natl Acad Sci 84:2024-2028 1987
 72. van Holten-Verzantvoort AT, Bijvoet OLM, Hermans J, Harinck HIJ, Elte JWF, Beex LVAM, Cleton FJ, Kroon HM, Vermey P, Neijt JP, Blijham G. Reduced morbidity from skeletal metastases in breast cancer patients during long-term bisphosphonate (APD) treatment. Lancet 8566:983-985 1987
 73. Cleton FJ, van Holten-Verzantvoort AT, Bijvoet OLM. Effect of long-term bisphosphonate treatment on morbidity due to bone metastases in breast cancer patients. Recent Results in Cancer Research 116:73-78 1989
 74. Burckhardt P, Thiebaud D, Perey L, von Fliedner V. Treatment of tumor-induced osteolysis by APD. Recent Results in Cancer Research 116:54-66 1989
 75. Bassani D, Sabatini M, Scanziani E, De Francesco L, Coccioli G, Guaitani A, Bartosek I. Bone invasion by Walker 256 carcinoma, line A in young and adult rats: effects of etidronate. Oncology 47:160-165 1990
 76. Sasaki A, Boyce BF, Story B, Wright KR, Chapman M, Boyce R, Mundy GR, Yoneda T. The bisphosphonate risedronate reduces metastatic human breast cancer burden in bone in nude mice. Can Res (in press) 1995
 77. Gurney H, Grill V, Martin TJ. Parathyroid hormone-related protein and response to pamidronate in tumour-induced hypercalcaemia. Lancet 341:1611-1613 1993
 78. Dodwell DJ, Abbas SK, Morton AR, Howell A. Parathyroid hormone-related protein⁽⁵⁰⁻⁶⁹⁾ and response to pamidronate therapy for tumour-induced hypercalcaemia. Eur J Cancer 27(12):1629-1633 1991
 79. Sato K, Yamakawa Y, Shizume K, Satoh T, Nohtomi K, Demura H, Akatsu T, Nagata N, Kasahara T, Ohkawa H, Ohsumi K. Passive immunization with anti-parathyroid hormone-related protein monoclonal antibody markedly prolongs survival time of hypercalcemic nude mice bearing transplanted human PTHrP-producing tumors. J Bone Min Res 8(7):849-860 1993

80. Engel LW, Young NA. Human breast carcinoma cells in continuous culture: a review. Can Res 38:4327-4339 1978
81. Furlanetto RW, DiCarlo JN. Somatomedin-C receptors and growth effects in human breast cells maintained in long term tissue culture. Can Res 44:2122-2128 1984
82. Lippman ME, Dickson RB, Gelmann EP, Rosen N, Knabbe C, Bates S, Bronzert D, Huff K, Kasid A. Growth regulation of human breast carcinoma occurs through regulated growth factor secretion. J Cell Biochem 35:1-16 1987
83. Paciotti GF and Tamarkin L. Interleukin-1 directly regulates hormone-dependent human breast cancer cell proliferation in vitro. Mol Endo 2:459-464 1988
84. Minne H, Rane F, Bellwinkel S, Ziegler R. The hypercalcemic syndrome in rats bearing the Walker carcinosarcoma 256. Acta Endocrinol 78:613-619 1975
85. DeMiguel F, Esbrit P. Purification of parathyroid hormone-related protein from the hypercalcemic rat strain of the rat Walker 256 carcinosarcoma. J Bone Min Res 6:suppl 1 A601 1991
86. Nakai M, Mundy GR, Williams PJ, Boyce B, Yoneda T. A synthetic antagonist to laminin inhibits the formation of osteolytic metastases by human melanoma cells in nude mice. Cancer Research 52:5395-5399 1992
87. Arguello F, Baggs RB, Frantz CN. A murine model of experimental metastasis to bone and bone marrow. Cancer Research 48:6878-6881 1988
88. Cailleau R, Yong R, Olive M, Reeves WJ. Breast tumor cell lines from pleural effusions. J Natl Cancer Inst 53:661-674, 1974
89. Stewart, A.F. Horst, R., Deftos, L.J., Cadman, E.C., Lang, R., and A.E. Broadus. Biochemical evaluation of patients with cancer-associated hypercalcemia: evidence for humoral and nonhumoral groups. N. Engl. J. Med. 303:1377-1383, 1980.
90. Pandian, M.R., Morgan, C.H., Carlton, E., and G.V. Segre. Modified immunoradiometric assay of parathyroid hormone-related protein: clinical application in the differential diagnosis of hypercalcemia. Clin. Chem. 38:282-288, 1992.
91. Budayr, A.A., Nissenson, R.A., Klein, R.F. Increased serum levels of a parathyroid hormone-like protein in malignancy-associated hypercalcemia. Ann. Int. Med. 111:807-812, 1989
92. Manolagas, S.C. Role of cytokines in bone resorption. Bone 17:63S-67S, 1995.
93. DeLaMata, J., H.L. Uy, T.A. Guise, B. Story, B.F. Boyce, G.R. Mundy and G.D. Roodman. IL-6 enhances hypercalcemia and bone resorption mediated by PTHrP *in vivo*. J. Clin. Invest. 95:2846-2852, 1995.
94. Rizzoli, R., J.H.M. Feyen, G. Grau, A. Wohlwend, A.P. Sappino and J-Ph. Bonjour. Regulation of parathyroid hormone-related protein production in a human lung squamous cell carcinoma line. Journal of Endocrinology 143:333-341, 1994.

95. Guise, T.A., S.D.Taylor, T. Yoneda, A.Sasaki, K. Wright, B.F. Boyce, J.M. Chirgwin and G.R. Mundy. PTHrP expression by breast cancer cells enhance osteolytic bone metastases *in vivo*. J. Bone Min. Res. 9(Suppl 1)33: S128a (Abstr.),1994.
96. Kiriyaama, T., M.T. Gillespie, J.A. Glatz, S. Fukumoto, J.M. Moseley and T.J. Martin. Transforming growth factor β stimulation of parathyroid hormone-related protein (PTHrP): a paracrine regulator? Mol. and Cell. Endocrinology 92:55-62, 1992.
97. Yoneda, T., P. Williams, C. Dunstan, J. Chavez, M. Niewolna and G.R. Mundy. Growth of metastatic cancer cells in bone is enhanced by bone-derived insulin-like growth factors. J Bone. Min. Res. 10(Suppl 1) P269a: S196a (Abstr.),1995.
98. Guise TA, Yin JJ, Taylor SD, Kumagai Y, Dallas M, Boyce BF, Yoneda T, Mundy GR. Evidence for a causal role of parathyroid hormone-related protein in breast cancer-mediated osteolysis. Journal of Clinical Investigation 1996;98(7):1544-1548.
99. Southby J, Murphy LM, Martin TJ, Gillespie MT 1996 Cell-specific and regulator-induced promoter usage and messenger ribonucleic acid splicing for parathyroid hormone-related protein. Endocrinology 137:1349-1357
100. Cailleau R, Yong R, Olive M, Reeves WJ 1974 Breast tumor cell lines from pleural effusions. J Natl Cancer Inst 53:661-674
100. Hortobagyi GN, Theriault RL, Porter L, Blayney D, Lipton A, Sinoff C, Wheeler H, Simeone JF, Seaman J, Knight RD, Heffernan M, Reitsma DJ 1996 Efficacy of pamidronate in reducing skeletal complications in patients with breast cancer and lytic bone metastases. N Engl J Med 335(24):1785-1791
101. Pfeilschifter J, Mundy GR 1987 Modulation of type β transforming growth factor activity in bone cultures by osteotropic hormones. Proc Natl Acad Sci USA 84:2024-2028
102. Zakalik D, Diep D, Hooks MA, Nissenson RA, Strewler GJ 1992 Transforming growth factor β increases stability of parathyroid hormone-related protein messenger RNA. J Bone Miner Res 7(Suppl 1):104A, S118
103. Soifer NE, Dee KE, Insogna KL, Burtis WJ, Matovcik LM, Wu TL, Milstone LM, Broadus AE, Philbrick WM, Stewart AF 1992 Parathyroid hormone-related protein. Evidence for secretion of a novel mid-region fragment by three different cell types. J Biol Chem 367:18236-18243
104. Orloff JJ, Soifer NE, Fodero JP, Dann P, Burtis WJ 1993 Accumulation of carboxyl-terminal fragments of parathyroid hormone-related protein in renal failure. Kidney International 43:1371-1376
105. Liu B, Amizuka N, Goltzman D, Rabbani 1995 Inhibition of processing of parathyroid hormone-related peptide by antisense furin: effect *in vitro* and *in vivo* on rat leydig (H-510) tumor cells. Int J Cancer 63:276-281
106. Bruzzaniti A, Goodge K, Jay P, Taviaux SA, Lam MH, Berta P, Martin TJ, Moseley JM, Gillespie MT 1996 C8, a new member of the convertase family Biochemical Journal 314:727-731
107. Orloff JJ, Reddy D, de Papp A, Yang KH, Soifer NE, Stewart AF 1994 Parathyroid hormone-related protein as a prohormone: posttranslational processing and receptor

interactions. Endocrine Reviews 15:40-60

108. Plawner LL, Philbrick WM, Burtis WJ, Broadus AE, Stewart AF 1995 Cell type-specific secretion of parathyroid hormone-related protein via the regulated versus the constitutive secretory pathway. J Biol Chem 270:14078-14084
109. Wu TL, Soifer NE, Burtis WJ, Milstone LM, Stewart AF. 1991 Glycosylation of parathyroid hormone-related peptide secreted by human epidermal keratinocytes. J Clin Endocrinol Metab 73:1002-1007
110. Orloff JJ, Kats Y, Urena P, Schipani E, Vasavada R, Philbrick WM, Behal Am Abou-Samra A-B, Segre GV, Jüppner H 1995 Further evidence for a novel receptor for amino-terminal parathyroid hormone-related protein on keratinocytes and squamous carcinoma cell lines. Endocrinology 136:3016-3023.

FIGURE 1

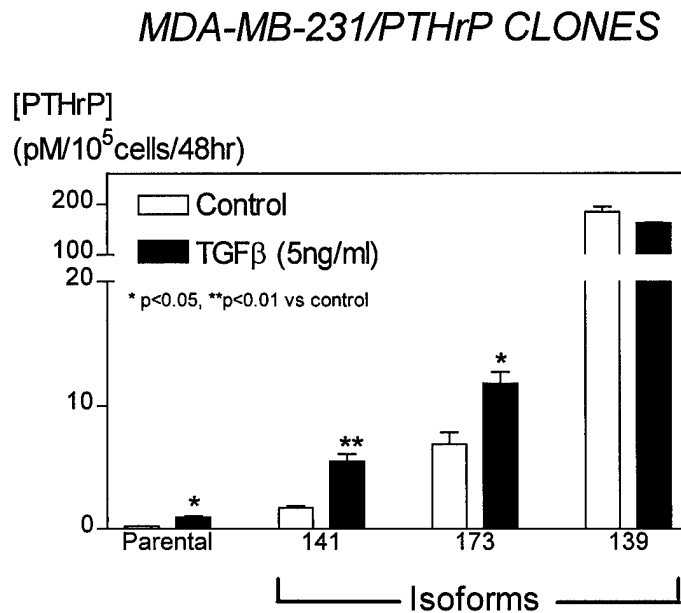


FIGURE 1: PTHrP secretion by parental MDA-MB-231 cells and representative clones MDA/PTHrP-(1-141), MDA/PTHrP-(1-173) and MDA/PTHrP-(1-139) in the basal state and in response to TGFβ. Respective cells were plated onto 48-well plates and grown to near confluence. Cells were washed and incubated with serum-free media in the presence or absence of TGFβ (5 ng/mL) for 48 hours. PTHrP concentrations in conditioned media were corrected for cell number. Values represent the mean ± SEM. N = 3 per group. Parental represent the untransfected and uncloned MDA-MB-231 cells while the representative transfectants are abbreviated with the respective isoform.

FIGURE 2

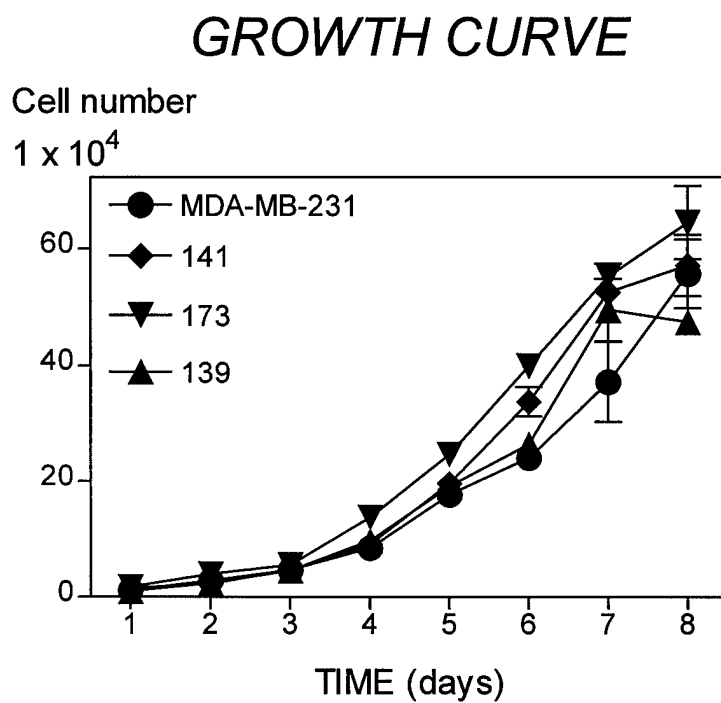
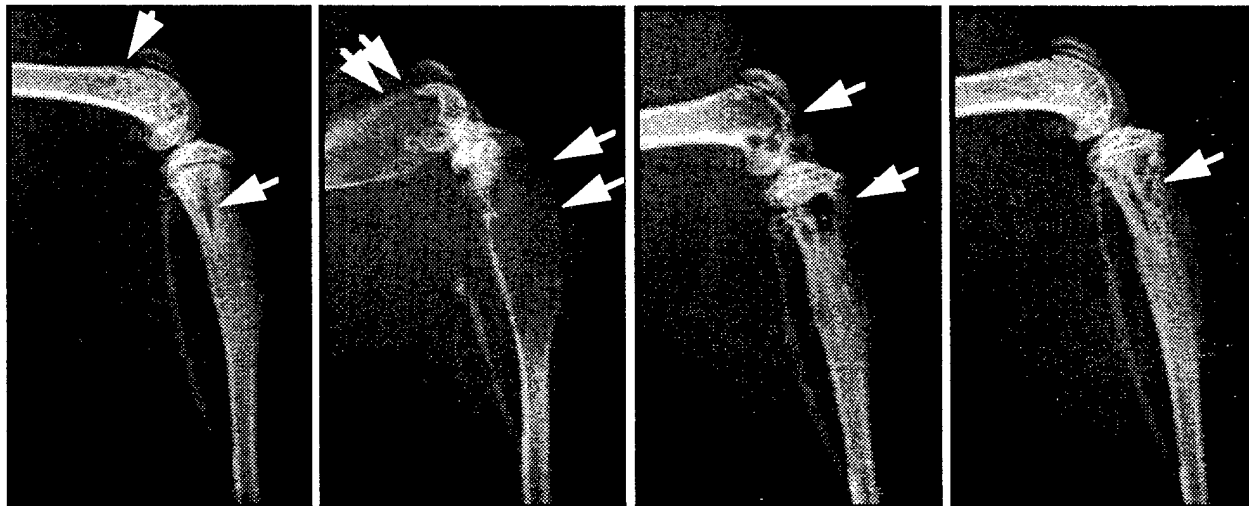


FIGURE 2: Growth rate of parental MDA-MB-231, MDA/PTHrP-(1-141), MDA/PTHrP-(1-173) and MDA/PTHrP-(1-139) cells. Respective cells were plated at a density of 10^4 cells/well in 10% FCS and counted daily. Values represent the mean \pm SEM. N = 3 per group. Parental represent the untransfected and uncloned MDA-MB-231 cells while the representative transfectants are abbreviated with the respective isoform.

FIGURE 3

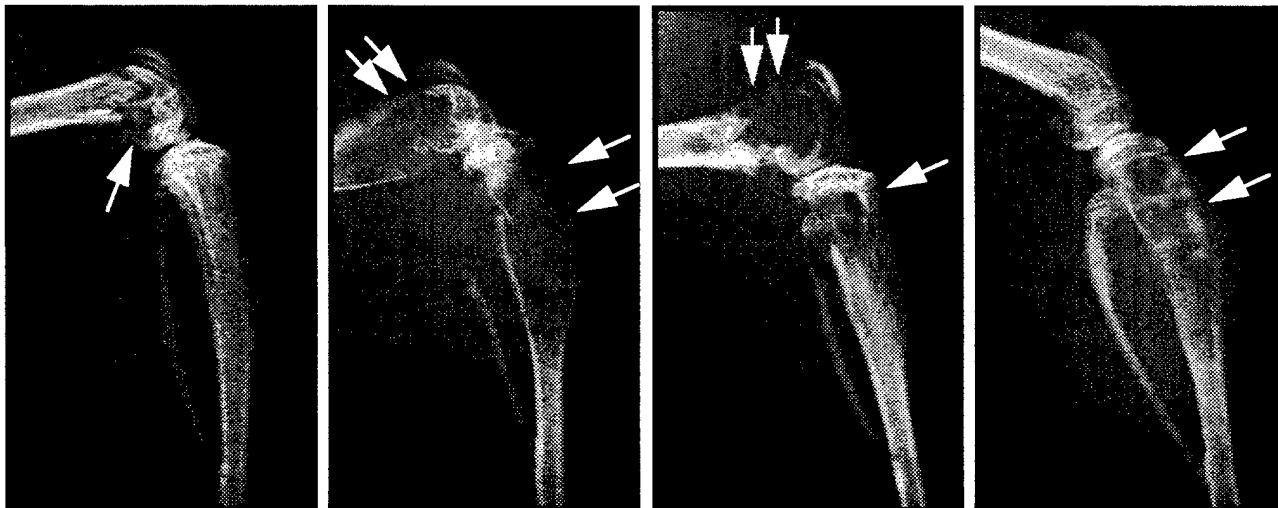


PARENTAL

139

141

173



PARENTAL
69d

139
56d

141
76d

173
85d

FIGURE 3: Representative radiographs of hindlimbs from mice bearing parental, MDA/PTHrP-(1-141), MDA/PTHrP-(1-173) and MDA/PTHrP-(1-139) tumors. Top Panel: Radiographs were taken 48 days after inoculation of tumor cells. Osteolytic lesions are indicated by the arrows. The most bone destruction is evident in the mice bearing MDA/PTHrP-(1-139). Bottom Panel: Radiographs were taken at the time of sacrifice as indicated. All groups overexpressing PTHrP, regardless of the isoform, demonstrated significant bone destruction at the time of sacrifice compared with mice bearing the parental MDA-MB-231 tumors. However, this bone destruction was earlier and more severe in the mice bearing the MDA/PTHrP-(1-139) tumors. Parental represent the untransfected and uncloned MDA-MB-231 cells while the representative transfectants are abbreviated with the respective isoform.

FIGURE 4

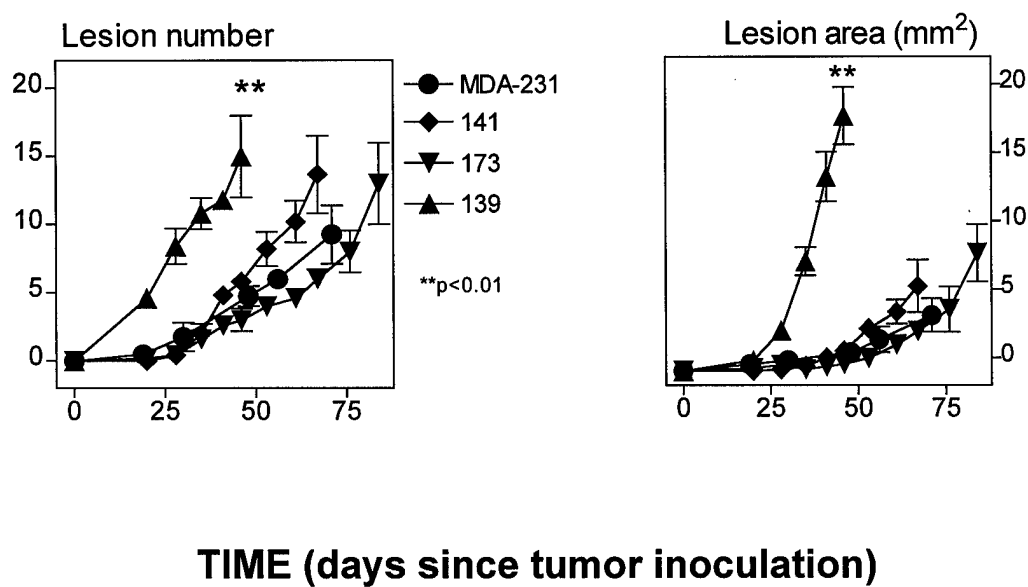


FIGURE 4: Osteolytic lesion number and area on radiographs from mice bearing parental MDA-MB-231, MDA/PTHrP-(1-141), MDA/PTHrP-(1-173) and MDA/PTHrP-(1-139) tumors as assessed by computerized image analysis. Respective tumor cells were inoculated on day 0. Lesion number and area was measured from long bones of fore- and hind limbs. Values represent the mean \pm SEM. N = 7 per group. Parental represent the untransfected and uncloned MDA-MB-231 cells while the representative transfectants are abbreviated with the respective isoform.

FIGURE 5

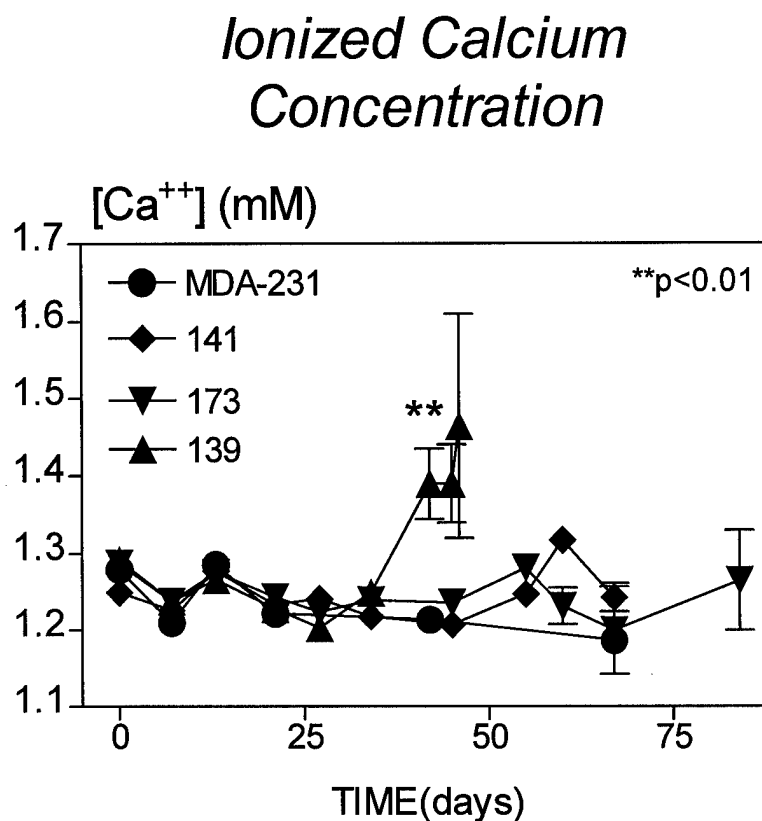


FIGURE 5: Whole blood ionized calcium concentrations in mice bearing parental MDA-MB-231, MDA/PTHrP-(1-141), MDA/PTHrP-(1-173) and MDA/PTHrP-(1-139) tumors. Calcium concentrations were significantly higher in mice bearing the MDA/PTHrP-(1-139) tumors compared with the other groups. Values represent the mean \pm SEM. N = 7 per group. Parental represent the untransfected and uncloned MDA-MB-231 cells while the representative transfectants are abbreviated with the respective isoform.

FIGURE 6

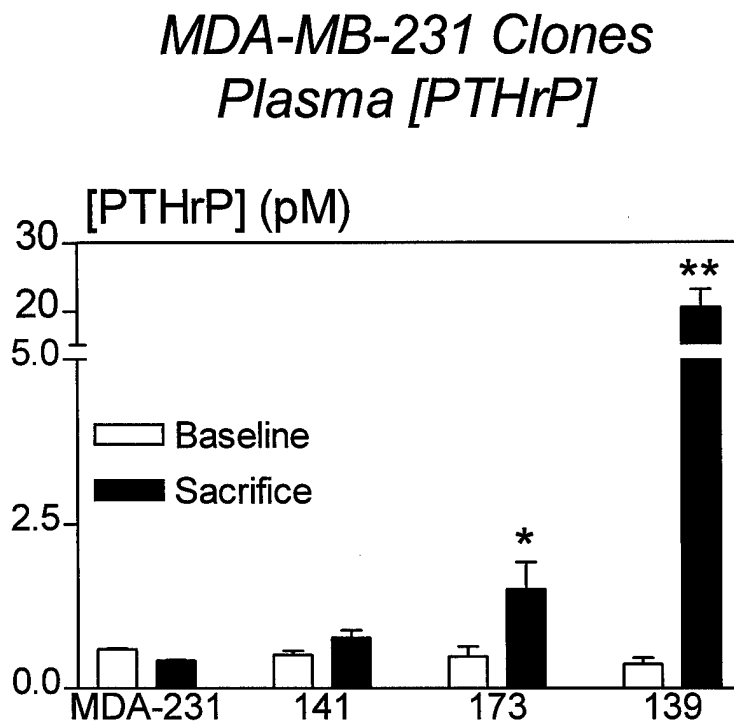


FIGURE 6: Plasma PTHrP concentrations in mice bearing parental MDA-MB-231, MDA/PTHrP-(1-141), MDA/PTHrP-(1-173) and MDA/PTHrP-(1-139) tumors. Plasma PTHrP concentrations at sacrifice were significantly higher than respective concentrations prior to tumor inoculation (baseline) in mice bearing MDA/PTHrP-(1-173) and MDA/PTHrP-(1-139) tumors. Values represent the mean \pm SEM. N = 7 per group. Parental represent the untransfected and uncloned MDA-MB-231 cells while the representative transfectants are abbreviated with the respective isoform.